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AUTOMATED DNA DIAGNOSTICS USING AN ELISA-BASED %%%OLIGONUCLEOTIDE%%%
%%LIGATION%% ASSAY

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ASSAY

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Automated DNA diagnostics using an ELISA-based oligonucleotide ligation assay

(DNA amplification/gene detection/genome mapping)

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Contributed by Leroy Hood, August 16, 1990

ABSTRACT DNA diagnostics, the detection of specific DNA sequences, will play an increasingly important role in medicine as the molecular basis of human disease is defined. Here, we demonstrate an automated, nonisotopic strategy for DNA diagnostics using amplification of target DNA segments by the polymerase chain reaction (PCR) and the discrimination of allelic sequence variants by a colorimetric oligonucleotide ligation assay (OLA). We have applied the automated PCR/OLA procedure to diagnosis of common genetic diseases, such as sickle cell anemia and cystic fibrosis ($\Delta F508$ mutation), and to genetic linkage mapping of gene segments in the human T-cell receptor β -chain locus. The automated PCR/OLA strategy provides a rapid system for diagnosis of genetic, malignant, and infectious diseases as well as a powerful approach to genetic linkage mapping of chromosomes and forensic DNA typing.

The study of DNA sequence variants in humans is playing an important role in diagnosis of genetic and malignant diseases (1, 2). The analysis of DNA polymorphisms also serves as the fundamental tool in attempts to construct genetic linkage maps (3, 4) and in forensic analyses (5, 6). Since the majority of DNA sequence variants and polymorphisms are single nucleotide substitutions (1, 2), diagnostic techniques must accurately discriminate single base changes.

Single base variations in DNA sequences can be detected by a variety of techniques including Southern blot analysis (7) for restriction fragment length polymorphisms, allele-specific oligonucleotide hybridization (8), denaturing gradient gel electrophoresis (9), chemical cleavage of mismatched heteroduplexes (10), conformational changes in single strands (11), and allele-specific priming of the polymerase chain reaction (PCR) (12-14). These techniques have several disadvantages for automating DNA diagnosis, which include the use of radioactivity, the requirement for various hybridization conditions, and the need for electrophoresis or centrifugation.

The analysis of DNA sequence variants has been greatly facilitated by the development of rapid methods to exponentially amplify specific DNA or RNA targets. Diagnostic targets can be amplified by PCR (15-17) or by other available methods (18-21). Amplification generates specific targets with high signal/noise ratios and permits the use of less sensitive nonisotopic reporters in DNA analysis.

An alternative strategy for DNA diagnosis, the oligonucleotide ligation assay (OLA), employs two adjacent oligonucleotides (20-mers), a 5' biotinylated probe (with its 3' end at the nucleotide to be assayed) and a 3' reporter probe (22-24). The two oligonucleotides are hybridized to target DNA and, if there is perfect complementarity, the enzyme

DNA ligase covalently joins the 5' biotinylated probe and the 3' reporter probe. If the probes and target are mismatched at their junction, a covalent bond is not formed. Capture of the 5' biotinylated probe on immobilized streptavidin and analysis for covalently linked 3' reporters determine the nature of the probe-target interaction (matched or mismatched). The ligase assay uses a standard set of conditions to distinguish all nucleotide mismatches, and product analysis does not require electrophoresis or centrifugation (22). In this report, we describe a strategy for automating DNA diagnosis that combines target amplification by PCR with a nonisotopic analysis of DNA sequence variants by OLA.

MATERIALS AND METHODS

Robotic Workstation. A Biomek 1000 workstation (Beckman) equipped with multipipet tools and a multibulk tool was used to perform all pipetting, aspirating, and washing procedures. The workstation has been modified with a solenoid to switch wash solutions during the ELISA. All reagents for sample processing were stored in sterile 96-minitube cassettes.

DNA Samples. DNA from humans with α_1 -antitrypsin, β -globin, and cystic fibrosis variants was obtained from F. Heijtmancik (Baylor University), from K. Tanaka (Harbor Hospital) and J. Korenberg (Cedar-Sinai Hospital), and from A. Osher and E. Hsu (Children's Hospital), respectively, and prepared as described (22). DNA for amplification of human T-cell receptor β -chain (TCR β) gene segments was obtained by gently scraping cells from the lining of the buccal cavity with a sterile toothpick. Buccal cells were dislodged into a minitube containing 10 μ l of sterile H₂O, covered with 75 μ l of mineral oil, and placed into a 96-minitube cassette for handling by the robotic workstation. Cells were lysed with 20 μ l of 0.1 M KOH and 0.1% Triton X-100 at 65°C for 20 min and neutralized with 20 μ l of 0.1 M HCl and 0.1% Triton X-100.

Oligonucleotides. Amplification primers and ligation probes were assembled by using standard phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer. Ligation probes were modified with a 5' biotin group as described (15) or chemically phosphorylated with 5' Phosphate-ON (Clontech) according to the manufacturer's directions. Modified probes were purified by reverse-phase high-performance liquid chromatography. Phosphorylated oligonucleotide probes (500 pmol) were labeled with dUTP-digoxigenin by mixing 100 mM potassium cacodylate, 2 mM CoCl₂, 200 μ M dithio-

Abbreviations: PCR, polymerase chain reaction; OLA, oligonucleotide ligation assay; TCR β , T-cell receptor β chain; CFTR, cystic fibrosis transmembrane conductance regulator; V, variable; D, diversity; J, joining; C, constant; STS, sequence-tagged site.

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threitol, 2.5 μ l of dUTP-digoxigenin (Boehringer Mannheim), and 2 μ l of adenosine triphosphate (40 μ M) with 70 units of terminal deoxynucleotidyltransferase (Collaborative Research) for 1 hr at 37°C. Free dUTP-digoxigenin was removed by two successive ethanol precipitations.

DNA Amplification. The robotic workstation was programmed to assemble PCR reagents [5 μ l containing 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM MgCl₂, 20 ng of bovine serum albumin per ml, the four deoxynucleotide triphosphates each at 400 μ M, 0.5 μ M amplification primers, 0.1% Triton X-100, and 0.05 unit of *Thermus aquaticus* DNA polymerase per well], genomic DNA (5 μ l at 2 ng/ μ l in sterile distilled H₂O containing 0.1% Triton X-100), and 70 μ l of light mineral oil in a flexible U-bottomed 96-well microtiter plate (Falcon). Genomic DNA samples were denatured at 93°C for 4 min and amplified by 40 cycles of 93°C for 30 sec, 55°C [cystic fibrosis transmembrane conductance regulator (CFTR) and TCR α constant (C α) gene segments] or 61°C (β -globin and α_1 -antitrypsin gene segments) for 45 sec, and 72°C for 90 sec in a microtiter plate thermal cycler (MJ Research, Watertown, MA). For amplification of TCR β gene segments, 15 μ l of PCR reagents (as described above) containing all six amplification primers, 15 μ l of the lysed buccal samples, and 70 μ l of mineral oil were added to a flexible microtiter plate. Targets were denatured at 93°C for 4 min and amplified by 20 cycles of 30 sec at 93°C, 45 sec at 61°C, and 90 sec at 72°C. Five microliters from these reaction mixtures was used to initiate a second round of amplification for each of the individual TCR β gene segments (40 cycles; 30 sec at 93°C, 45 sec at 61°C, and 90 sec at 72°C).

Ligation Assays. Ligation reaction mixtures were assembled by the robotic workstation. Forty-five microliters of 0.25 M NaOH containing 0.1% Triton X-100 was added to amplified DNA samples. Ligation probes (200 fmol each) in 10 μ l of 2 \times ligase buffer (100 mM Tris-HCl, pH 7.5/20 mM MgCl₂/2 mM spermidine/2 mM adenosine triphosphate/10 mM dithiothreitol) and 50% formamide were added to a U-bottomed 96-well microtiter plate. DNA samples were neutralized with 45 μ l of 0.25 M HCl and six 10- μ l aliquots were added to the microtiter plate containing the ligation probes. Samples were covered with 70 μ l of mineral oil, denatured at 93°C for 2 min, cooled, and returned to the workstation for the addition of 5 μ l of T4 DNA ligase (5 units/ml) (Amersham) in 1 \times ligase buffer. Ligations were done at room temperature (RT) for 15 min. Reactions were stopped by adding 10 μ l of 0.25 M NaOH per well and, after 2 min at RT, 4 μ l of 3 M sodium acetate (pH 6.5) per well. Samples were transferred to a 96-well flat-bottomed microtiter plate (Falcon) coated with streptavidin [60 μ l of streptavidin (100 μ g/ml) or avidin (100 μ g/ml) (Vector Laboratories) for 1 hr at 37°C] and blocked 20 min (RT) before use with 200 μ l of 100 mM Tris-HCl, pH 7.5/150 mM NaCl/0.05% Tween 20 (buffer A) per well with 0.5% dry milk and 100 μ g of salmon sperm DNA per ml. Biotinylated probes were captured at RT for 30 min, and the plate was washed twice with 0.01 M NaOH and 0.05% Tween 20 and once with buffer A. Thirty microliters of anti-digoxigenin antibodies (diluted 1:1000; Boehringer Mannheim) in buffer A with 0.5% dry milk was added to each microtiter well. Plates were incubated 30 min (RT) and washed six times with buffer A. Substrate (30 μ l of BRL ELISA amplification system per well) was added, the plates were incubated 15 min (RT), and 30 μ l of amplifer was added. Spectrophotometric absorbances were taken at 490 nm by a Bio-Tek (Burlington, VT) plate reader and absorbances were directly entered into an IBM-XT computer.

Linkage Analysis. Observed haplotype frequencies were calculated for genetic linkage analysis of TCR β gene segments with a myriad haplotype program (25). The probability of linkage disequilibrium was calculated based on the χ^2 distribution of the *Q* statistic described by Hedrick *et al.* (26).

RESULTS

The Automated PCR/OLA Strategy. Our strategy for automated gene analysis is shown in Fig. 1. A Biomek 1000 robotic workstation was used to (i) prepare targets and assemble reagents for DNA amplification, (ii) mix and ligate 5' biotinylated probes and 3' digoxigenin-labeled reporter probes on amplified DNA targets using T4 DNA ligase, (iii) capture 5' biotinylated probes on streptavidin-coated microtiter plates, (iv) wash plates, and (v) detect the digoxigenin reporter coupled to biotin-labeled probes by an ELISA. Altogether, processing time for 96 samples from entry to computer read-out takes <7 hr. Overnight amplification permits processing of ligation assays from 192 DNA samples in a single day (1200 reactions, triplicates for two alleles).

Amplification Primers and Ligation Probes. A panel of amplification primers and ligation probes for known sequence variants in human DNA have been synthesized (Table 1). Two sets of probes detect mutations that cause common genetic diseases in homozygous individuals, sickle cell anemia and CF (27, 28). Another set detects a common mutation in the α_1 -antitrypsin gene that, in homozygous individuals, leads to a predisposition for cirrhosis of the liver in childhood and emphysema in adults (29). The remaining probes detect

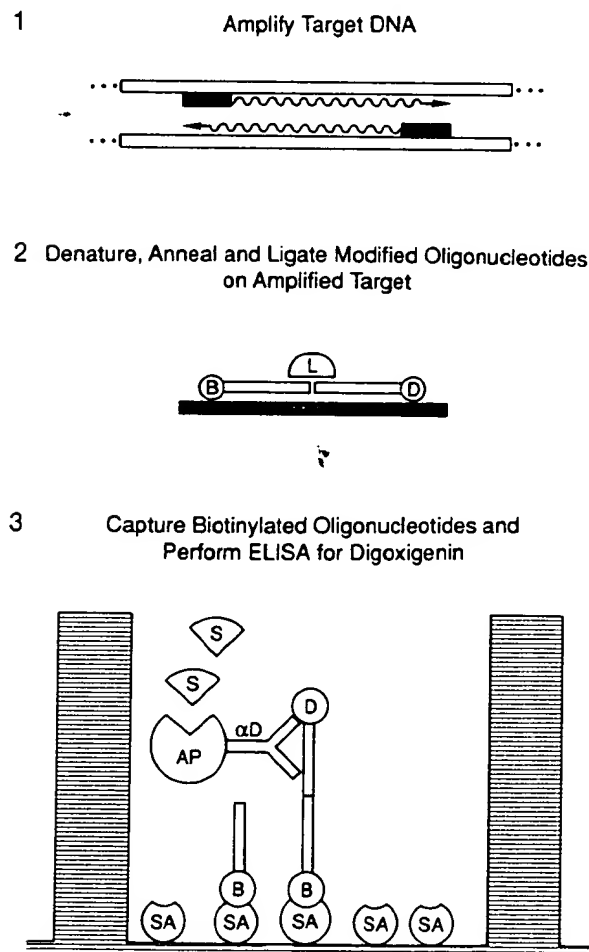


FIG. 1. Schematic diagram of the steps in the automated PCR/OLA procedure performed with a robotic workstation. The assay contains three steps: 1. DNA target amplification; 2. analysis of target nucleotide sequences with biotin (B)-labeled and digoxigenin (D)-labeled oligonucleotide probes and T4 DNA ligase (L); 3. capture of the biotin (B)-labeled probes on streptavidin (SA)-coated microtiter wells and analysis for covalently linked digoxigenin (D) by using an ELISA procedure with alkaline phosphatase (AP)-conjugated anti-digoxigenin (α D) antibodies and a substrate (S).

Table 1. Nucleotide sequence of the amplification primers and ligation probes used in automated DNA analysis

Genomic region amplified	Amplification primers	Ligation probes		Target detected by ligation probes
		Biotin-labeled probe	Reporter-labeled probe	
β -Globin	CAACTTCATCCACGTTACCTTGCC AGGGCAGGAGCCAGGGCTGGG	1. B-ATGGTGCACCTGACTCCTGA 2. B-ATGGTGCACCTGACTCCTGT	pGGAGAAGTCTGCCGTTACTG-D	1. β A 2. β S
α_1 -Antitrypsin	TCAGCCTTACAACGTGTCTCTGCTT GTATGGCCTCTAAAAACATGGCCCC	1. B-GGCTGTGCTGACCATCGACG 2. B-GGCTGTGCTGACCATCGACA	pAGAAAGGGACTGAAGCTGCT-D	1. M 2. Z
CFTR	CAGTGAAGAATGGCATTCTGTT GGCATGCTTTGATGACGCTTCTG	1. B-ATTAAGAAAAATATCATCTT 2. B-ACCATTAAGAAAAATATCAT	pTGGTGTTCCTATGATGAAT-D	1. Non-F508 2. Δ F508
C_α	CCTTGAAGCTGGGAGTGG GAGCTAAGAGAGCCGTACTGG	1. B-GAAACGAAGAACTGAGGCCA 2. B-GAAACGAAGAACTGAGGCC	pCAGAGCTAATGAGTGAGGAAGA-D	1. C_α 3A 2. C_α 3B
V_{β} 6.71	AAGGGAAAGGATGTAGAG CTGGCACAGAGATACACGGCC	1. B-TTTACTGGTACCGACAGAGC 2. B-TTTACTGGTACCGACAGAGG	pCTGGGGCAGGGCCTGGAGTT-D	1. V_{β} 6.71A 2. V_{β} 6.71B
V_{β} 6.72	AAGGGAAAGGATGTAGAG CTGGCACAGAGATACACGGCC	1. B-TCTGCAGAGAGGACTGGGG 2. B-TCTGCAGAGAGGACTGGGA	pATCCGTCTCCACTCTGACGA-D	1. V_{β} 6.72A 2. V_{β} 6.72B
V_{β} 1	GAGTCACACAAACCCAAAGCACCT GCTGCTGGCACAGAAATACAAAGCT	1. B-AGGCCTCCAGTTCCTCATTAC 2. B-AGGCCTCCAGTTCCTCATTAC	pTATTATAATGGAGAAGAGAGAGCA-D	1. V_{β} 1A 2. V_{β} 1B
C_β	CATTATGGTCCTTCCCGG AGCTCCACGTGCTCGGGT	1. B-ACCAGGACCAGACAGCTCTC 2. B-ACCAGGACCAGACAGCTCTT	pAGAGCAACCTAGCCCCATTAC-D	1. C_β 3A 2. C_β 3B

Ligation reactions were performed with a mixture of a biotin-labeled and reporter-labeled probe for each specific allele.

polymorphisms in the human TCR β and TCR α loci (refs. 30 and 31; C. Whitehurst, P. Charmley, L.H., and D.A.N., unpublished data). Most of these probes detect single nucleotide substitutions in a specific DNA target. However, one set of probes detects a 3-base-pair (bp) deletion in the gene encoding CFTR (28) and represents a model for the detection of sequence deletions by OLA.

Analysis of DNA Sequence Variants. As a model for DNA diagnosis by the PCR/OLA procedure, we obtained genomic DNAs from 32 individuals of known genotype. The robotic workstation was used to assemble PCR reagents and genomic DNA samples in a 96-well microtiter plate. After amplification, ligations were performed in triplicate for each allele, and the immobilized probes were analyzed for the presence of digoxigenin. An example of a microtiter plate obtained from this process is shown in Fig. 2. Amplified targets from homozygous and heterozygous individuals for the indicated nucleotide substitutions (β -globin, α_1 -antitrypsin, and TCR C_α) or deletion (CFTR) were used. The assay clearly identifies which alleles 1 and/or 2 (Table 1) were present in each of the amplified samples (Fig. 2). Fig. 3 shows the mean absorbances obtained from ligation assays on amplified DNA targets from eight different individuals for each of the analyzed gene segments (32 individuals altogether). Mean absorbances from different individuals ranged from 0.38 to 1.17. We have found that mean absorbances from the ligation assays reflect the amount of target present in an amplified DNA sample. In this regard, the colorimetric assay is quite sensitive and can detect 3 fmol of ligated product (data not shown). The high signal/noise ratios (10:1–200:1) obtained with this procedure also permit simple data processing to define the genotype of an amplified DNA sample by calcu-

lating the ratio of the mean absorbance for each allele in the ligation assay. Furthermore, since the outcome of the PCR/OLA procedure is based on the mean absorbance of triplicate ligation reactions, the chance of error arising from spurious false-negative or false-positive wells is also minimized (false-negative or false-positive wells < 0.2% in 4000 reactions; data not shown).

Genetic Linkage Analysis of TCR β Genes. The automated PCR/OLA protocol has been extended to include the preparation of DNA samples by the robotic workstation. Amplified DNA targets from human buccal samples were used to determine the frequency and genetic linkage of four DNA sequence polymorphisms in the human TCR β locus as shown in Fig. 4. The human TCR β locus is composed of several gene segments, variable (V), diversity (D), end joining (J), and constant (C) genes, which span >600 kilobases (kb) of DNA (Fig. 4) (32, 33). Using data obtained from the automated PCR/OLA procedure on these 96 samples, we found that two V_{β} 6.7 polymorphisms were in complete linkage disequilibrium ($P < 10^{-14}$). This finding was not surprising since these variants are separated by a small physical distance (100 bp). Although the exact location of the V_{β} 6.7 gene segment in the TCR β locus is not known, analysis of available cosmid and YAC clones by gene-specific PCR suggests that V_{β} 6.7 is probably located 5' to the V_{β} 1 gene segment. The three TCR polymorphisms (V_{β} 6.7, V_{β} 1, and C_β), physically spanning at least 600 kb, appeared to be in linkage equilibrium with one another. Indeed, the expected haplotype frequencies calculated assuming linkage equilibrium were very close to those observed ($P < 0.81$) (Table 2). These findings confirm those recently reported in a study of TCR polymorphisms detected as restriction fragment length polymorphisms and may sug-

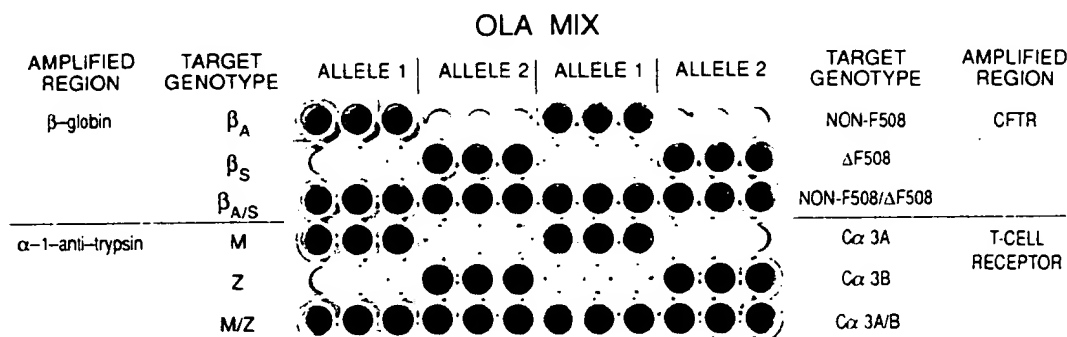


FIG. 2. Amplified DNA targets obtained from genomic DNA samples were analyzed in triplicate by using the indicated combinations of ligation probes (alleles 1 and 2 as described in Table 1) for each specified gene segment. Wells containing digoxigenin form a magenta-colored product and indicate complementarity between the ligation probes and amplified DNA target.

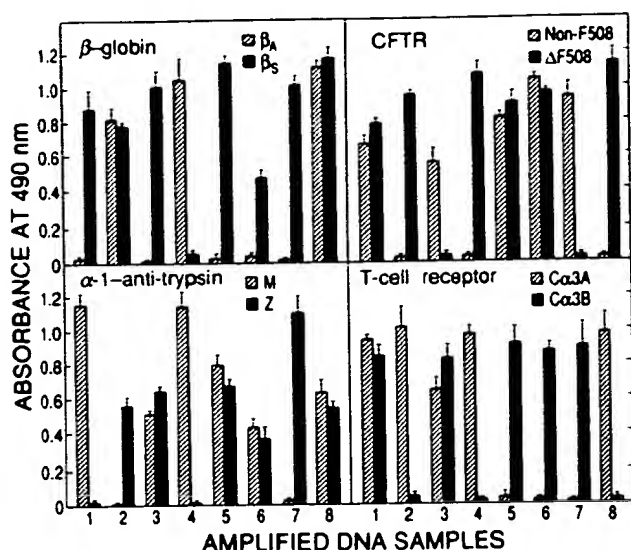


FIG. 3. Mean spectrophotometric absorbances (± 1 SD) from triplicate ligation reactions performed by the automated PCR/OLA procedure on amplified DNA samples obtained from eight donors for each gene analyzed (32 DNA samples total).

gest that hot spots of recombination exist in the TCR β locus (34).

DISCUSSION

Automated analysis of DNA polymorphisms and variants by PCR/OLA has many advantages over existing approaches to DNA diagnostics. Small numbers of cells (cheek scraping) or DNA samples (10 ng) are sufficient for analysis. Only small fragments of DNA (a few hundred base pairs) are required. Therefore, partially degraded DNA is still useful. The reagents are stable and easily obtained, and nonisotopic reporter groups are used. The entire assay is performed in microtiter wells, thus avoiding the use of centrifugation or electrophoresis. The assay yields high signal/noise ratios and a simple readout that is easily transferred to a computer for storage and analysis: no measurements of DNA fragment sizes are necessary. All of the tested sequence variants (nucleotide transitions and transversions, and a deletion) could be discriminated by OLA using a standard set of conditions. The initial PCR amplification facilitates the discrimination of polymorphisms in individual members of a multigene family (e.g., the TCR $V_{\beta 6.7}$ gene segment is one of

Table 2. TCR haplotypes

Haplotype	Observed	Expected
$V_{\beta 6.7}$ $V_{\beta 1}$ C_{β}		
AAA	64	69
AAB	40	36
ABA	11	14
ABB	9	7
BAA	40	36
BAB	17	19
BBA	6	7
BBB	5	4

Expected haplotypes were calculated assuming random allelic association—e.g., $AAA = 0.66 \times 0.83 \times 0.66 \times 192 = 69$.

nine highly similar members of the $V_{\beta 6}$ subfamily). The two successive levels of sequence discrimination, PCR and then OLA, enhance signal/noise ratios and reduce the likelihood of error, particularly in the analysis of polymorphisms in multigene families. The steps in the assay are automatable, eliminating the need for human intervention (and possible mistakes) in a tedious and repetitious process. With automation, high throughput is possible. At present, we can process 1200 ligation reactions per day with a single operator and robotic workstation, and, in the near future, further automation with a robotic arm will permit processing of 6000 reactions per day.

The automated PCR/OLA assay can be applied in many different basic research and clinical areas. Genetic diseases fall into several different categories including the common and widespread mutations of sickle cell disease, α_1 -antitrypsin or CF, and newly arising spontaneous mutations such as Lesch-Nyhan disease (35). Clearly, PCR/OLA facilitates the analysis of the common mutations, either in screening at-risk members of families with diseases or for more general carrier screening purposes. Rapid techniques are being developed to identify the sequence variations of newly arising mutations (35, 36). Once identified, the combined PCR/OLA procedure can be used to follow the inheritance of these specific mutations in affected families. Many genes cause a predisposition toward disease. This is true of the α_1 -antitrypsin mutation described above. Recently, it has been demonstrated that certain TCR and HLA haplotypes may predispose humans to certain autoimmune diseases such as multiple sclerosis (37–39). Therapeutic strategies are being developed to circumvent these predispositions (40–42). Therefore, automated screening may be useful in the near future to identify the genes associated with disease predis-

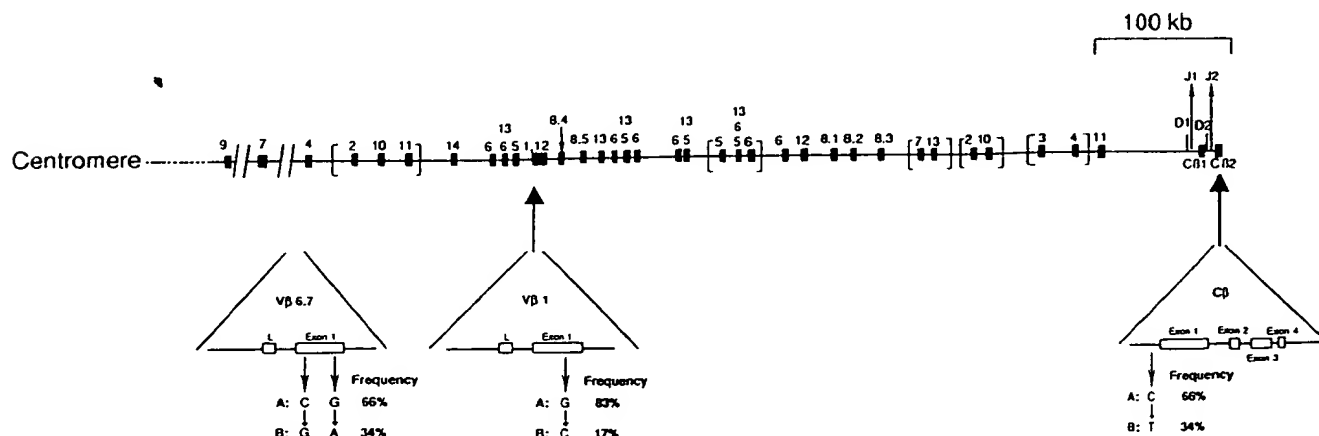


FIG. 4. Schematic diagram of the human TCR β locus giving the relative order of the V, D, J, and C gene segments. DNA polymorphisms in three indicated gene segments were analyzed in 96 individuals. Their location, where known, is shown (arrow up). The nucleotide substitutions analyzed and the frequency for each variant in these samples are shown.

positions in which some form of preventive therapy can be initiated.

The automated PCR/OLA procedure provides a powerful approach to high-resolution genetic linkage mapping of the human genome or other complex genomes. For this approach, sequence-tagged sites (STSs) (43) from specific chromosomal regions (e.g., the TCR β locus) or from a specific chromosome (e.g., STSs obtained from random clones of a flow-sorted chromosome library) would be scanned for internal DNA sequence polymorphisms (9–11) to obtain a set of polymorphic STSs. Once acquired, polymorphic STSs can be rapidly ordered by analysis of large multigeneration families or by single-sperm typing (44, 45) using the automated PCR/OLA system.

The availability of human polymorphic STSs will also provide a set of markers for automated forensic typing. For example, with a set of maximally informative biallelic markers (50:50 distribution in random mating populations) from each of the 22 human autosomes, the probability that two individuals would have identical DNA fingerprints—i.e., the same set of the 44 alleles—is ≈ 1 in 10^{10} . The automated PCR/OLA procedure eliminates most of the limitations associated with forensic typing by conventional Southern blot analysis (e.g., the measurement of DNA fragment sizes, the requirement for high quality DNA, and the use of radioisotopes).

Other applications for automated DNA diagnosis by the PCR/OLA procedure include HLA typing, the analysis of recessive or dominant oncogenes, and the identification of infectious pathogens. The use of commercially available thermostable ligases and automated ligation amplification reactions in the direct detection of single copy genes can also be explored. Moreover, multiple nonisotopic reporter groups may be developed that will be simultaneously analyzed in a single microtiter well. This raises the possibility of multiplexing the OLA procedure to the point where initially both alleles can be analyzed together and eventually multiple biallelic loci can be typed in a single well. These and other improvements, such as a single instrument to perform the entire analysis, will greatly increase the throughput and potential applications of automated DNA diagnostics.

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